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SYNTHESIS OF NEW STEROID HAPTENS FOR RADIOIMMUNOASSAY—VIII. DEVELOPMENT AND VALIDATION OF A SPECIFIC RADIOIMMUNOASSAY FOR SERUM 5α-ANDROSTANE-3α,17β-DIOL 17-GLUCURONIDE

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Summary—5α-Androstane-3α,17β-diol glucuronide (androstanediol-G) is a dihydrotestosterone metabolite whose serum levels are elevated in hirsute women. Current assay methods do not distinguish between the two androstanediol-G isomers, androstanediol 3-G and androstanediol 17-G. Since the production of these isomers may be influenced by different factors, we have developed a specific radioimmunoassay for androstanediol 17G. The antibody was raised against 5α-androstane-3α,17β-diol 17-G conjugated to bovine serum albumin (BSA). [9,11-^{3}H]5α-Androstane-3α,17β-diol 17-G was used for determination of procedural losses and as the labeled ligand in the assay. Unlabeled androstanediol 17-G was used as assay standard.

Serum levels of total androstanediol-G, androstanediol 3-G and androstanediol 17-G were measured in 8 normal men. Total androstanediol-G levels were 16.5 ± 5.2 nmol/l, androstanediol 17-G levels were 12.9 ± 5 nmol/l, and androstanediol 3-G levels were 3.3 ± 1.8 nmol/l. 77 ± 13% of total androstanediol-G was androstanediol 17-G.

These results confirm our previous findings that androstanediol 17-G is the predominant androstanediol-G isomer in human serum and suggests that 5α-dihydrotestosterone (DHT) is preferentially metabolized to androstanediol 17-G.

INTRODUCTION

Levels of 5α-androstane-3α,17β-diol glucuronide (androstanediol-G) are elevated in the plasma of women with idiopathic hirsutism [1] and lowered in men with disorders of androgen action [2]. For these reasons it has been suggested that androstanediol-G might serve as a marker for androgen action in androgen target tissues. Androstanediol-G can be conjugated as either the 3- or 17-glucuronide. We have recently established [3, 4] that androstanediol 17-G is the predominant form of androstanediol-G in serum. Present assay methods for serum androstanediol-G involve an enzymatic hydrolysis of the glucuronide followed by radioimmunoassay (RIA) of the unconjugated androstanediol. These methods are cumbersome, yield variable results due to incomplete enzymatic hydrolysis [5], and do not distinguish between the levels of the 3- and 17-glucuronide isomers. An RIA for androstanediol 17-G without an enzymatic hydrolysis is highly desirable. In addition, the levels of total androstanediol-G, androstanediol 3-G and androstanediol 17-G in serum of normal men are presented.

EXPERIMENTAL

Antibody Development

The antibody, labeled ligand, and standard were developed at the Southwest Foundation for Biomedical Research.

All solvents and reagents were purchased and purified as previously reported [6]. *N,N'*-Carbonyldiimidazole was obtained from Aldrich Chemical Co. Inc., Milwaukee, Wis. [9, 11-^{3}H]Androstanediol 17-G was prepared from the 9,11-unsaturated steroid substrate [7] by catalytic tritiation carried out at Dupont New England Nuclear Research Products, Boston, Mass. Base hydrolysis of the tritiated product gave [9,11-^{3}H]androstanediol 17-G with a specific activity of 54.9 Ci/mmol. Testosterone glucuronide and androsterone glucuronide were purchased from Sigma Chemical Co., St Louis, Mo. Androstanediol 17-G, androstanediol 3-G, and 17β-hydroxy-5α-androstan-3-one 17β-D-glucuronide were prepared by base hydrolysis of the corresponding authentic

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5α-androstane-3α,17β-diol 17-glucuronide triacetylmethyl ester (GAME), 5α-androstane-3α,17β-diol 3-GAME and 17β-hydroxy-5α-androstan-3-one 17-GAME described earlier [8].

Preparation of the steroid–bovine serum albumin (BSA) conjugate

Androstanediol 17-G (270 mg) was coupled to BSA (803 mg) by the procedure of Axen [9] using *N,N'*-carbonyldiimidazole [10]. Determination of free amino groups in the conjugate by a quantitative ninhydrin procedure [11] established that 26 mol of steroid were bound to 1 mol of BSA.

Immunization procedure and collection of the antibody

Three female New Zealand white rabbits, 4 months old, were used for immunization with the conjugate. The injection and the bleeding schedules were as reported [6] with the only exception that Freund's incomplete adjuvant was used in the preparation of the immunogenic material for injection.

Determination of cross-reactivity

An androstanediol 17-G reference curve was constructed with 0, 25, 50, 100, 250, 500 or 1000 pg androstanediol 17-G standard per tube. The cross-reacting steroid curves were constructed with 0.5, 1, 10, 20, 40 ng steroid per tube. The standard and cross-reacting compounds were prepared from stock solutions of unlabeled steroids in absolute ethanol (100 mg/ml). The tritium labeled glucuronide derivative was prepared in assay buffer at a concentration of 200 pg/ml. The antiserum was prepared in BSA assay buffer (1 g BSA/1000 ml 0.1 M sodium phosphate buffered saline pH 7). To all standard and cross-reacting tubes, antibody and 50 pg labeled steroid were added. These were mixed on a vortex mixer and allowed to incubate at 4°C overnight. After addition of 0.2 ml gamma globulin dextran-coated charcoal (1.2 g charcoal, 0.12 g dextran, 0.2 g human gamma globulin, 200 ml deionized water), each tube was again mixed and placed in a cold room at 4°C for 20 min. The samples were then centrifuged at 2500 rpm and the supernatant activity was counted to a relative standard error of less than 2% in a Beckman LS7500 liquid scintillation counter following addition of 6 ml scintillation cocktail (ScintiVerse, Fisher Scientific).

Measurement of androstanediol-G isomers in serum of normal men—RIAs

Development of the serum androstanediol 17-G assay and measurement of serum total androstanediol-G, androstanediol 3-G and androstanediol 17-G levels were done at Dalhousie University.

Androstanediol 17-G assay

Serum androstanediol 17-G was measured by RIA in ether-extracted serum followed by separation of the androgen conjugates by high-performance liquid chromatography (HPLC).

[9,11-^{3}H]Androstanediol 17-G tracer was used for both recovery determination and the labeled ligand in the RIA. For serum sample preparation, 2000 cpm of tritiated androstanediol 17-G were added to 500 μl serum. Unconjugated steroids were removed by extraction with diethyl ether (2 extractions using 3.5 ml ether per extraction) and the remaining aqueous phase was applied to an equilibrated (10 ml methanol followed by 10 ml water) C_{18} sample preparatory column (SepPak, Waters Scientific, Mississauga, Ontario) to remove serum proteins. The column was washed with 20 ml water and the androgen conjugate fraction was eluted from the column in 3 ml methanol. The sample was evaporated to dryness under vacuum (Savant Speed Vac., Emerston Instruments, Scarborough, Ontario) and reconstituted in 100 μl methanol. Androstanediol 17-G was separated from other androgen conjugates by reverse phase HPLC [4], using an isocratic mobile phase of 35:65, acetonitrile: 0.1% trifluoroacetic acid in water. Under these conditions, androgen sulfates eluted in the first 6 min. Androstanediol 17-G eluted as a single peak with a retention time of 9–12 min. This fraction was collected, evaporated to dryness, and reconstituted in 600 μl pH 7.5 phosphate buffered saline–0.1% gelatin (assay buffer). A 150 μl aliquot was taken for determination of procedural losses. For the assay, 200 μl sample was added to 100 μl of assay buffer. Androstanediol 17-G was used as assay standard. Dilutions of assay standard were brought up to 300 μl with assay buffer. Assay buffer (100 μl) containing 8000–10,000 cpm [^{3}H]androstanediol 17-G and 100 μl of a 1:2000 dilution of antibody (final tube dilution of 1:10,000) was added and the assay incubated at 4°C for 16–20 h. Unbound ligand was separated from bound ligand by dextran-coated charcoal prepared as a 0.1% dextran (70,000 mol. wt, Sigma, St Louis, Mo.): 0.5% activated charcoal (neutralized, Sigma, St Louis, Mo.) suspension in phosphate buffered saline pH 7.4. Supernatant radioactivity was determined in an LKB model 1211 RackBeta scintillation counter following the addition of 5 mls of scintillation cocktail (ScintiVerse, Fisher Scientific). All samples were assayed in duplicate. The RIA data were analyzed by a computer program that used weighted regression of the logit–log-transformed data. The results were corrected for procedural losses.

Androstanediol 3-G assay

Serum androstanediol 3-G was measured as previously described [4]. Briefly, 2000 cpm [^{3}H]androstanediol 3-G was added to 500 μl serum. Unconjugated steroids were removed by ether extraction (2 × 3.5 ml) and proteins were removed by chromatography, using a C_{18} preparatory column (Sep-Pak, Waters Scientific, Mississauga, Ontario). The androgen glucuronides obtained were separated by

HPLC using the same procedure as described for androstanediol 17-G.

The antibody used in the RIA was raised against androstanediol 3-G conjugated to BSA through the glucuronide moiety (0.3% cross-reactivity with androstanediol 17-G). [9,11-^{3}H]Androstanediol 3-G (produced by the same procedure as for androstanediol 17-G) was used for determination of procedural losses, and as the labeled ligand. Androstanediol 3-G was used as assay standard.

Total androstanediol-G assay

[^{3}H]Androstanediol 17-G (2000 cpm) was added to 500 μl serum. The serum was then extracted twice with 3.5 ml ether and the aqueous phase was adjusted to pH 5 with 0.2 M acetic acid. Hydrolysis was performed at 45°C for 18 h following the addition of 20,000 units of β-glucuronidase in 100 μl of 0.1 M sodium acetate buffer (pH 5). The liberated steroids were extracted with ether (2 × 4 ml), evaporated to dryness, reconstituted in 200 μl 70% iso-octane: 30% benzene and added to ethylene glycol:celite columns (0.75 ml ethylene glycol/1.5 g baked celite). The columns were washed with 12 ml of 70% iso-octane:30% benzene. The androstanediol fraction was then collected with 7 mls of 40% iso-octane:60% benzene. This fraction was evaporated to dryness and the sample reconstituted in 600 μl assay buffer (same as androstanediol 17-G assay buffer). Recovery was determined on 150 μl of sample and 2 × 200 μl aliquots were taken for RIA analysis. The RIA procedure was as described previously [4].

Assay calibrations. Androstanediol 3-G and androstanediol 17-G assays were calibrated against the standards by hydrolyzing the androstanediol 3-G and androstanediol 17-G and measuring the liberated A-diol by RIA.

Subjects. Total androstanediol-G, androstanediol 3-G and androstanediol 17-G levels were independently measured in the same serum samples from 8 normal healthy men, aged 21–45 yr who were not on any medications known to affect serum hormone levels.

Statistics. Serum steroid levels are presented as mean ± SD. The measured total androstanediol-G levels and the sum of the levels of the two isomers were compared by the paired Student's *t*-test.

RESULTS

Antibody development

Approximately 5 months following immunization all three rabbits produced antisera with high titers. Titer determinations were done on plasma of each rabbit following their monthly bleeding. The titer was determined from the ability of the antibody to bind a constant amount (50 pg) of the labeled steroid as compared to plasma collected prior to the primary injection. Each plasma sample was assessed at three different concentrations of antibody in order to determine the 50% binding level. Plasma from rabbit X-919 (7-19-89) with a titer of 1:10,000 was selected for complete characterization. The binding affinity constant as determined by a Scatchard plot [12] was found to be $K_a = 1.5 \times 10^{10}$ l/mol. The percent cross-reaction of the antibody was determined using the method of Abraham [13]. A standard curve was established over the desired working range and compared to the inhibition curves of pertinent androgen glucuronides and other steroids in normal circulation. The cross-reactivity data is presented in Table 1. Among the androgen glucuronides, androstanediol 3-G (5.86%) and dihydrotestosterone-G (1.75%) exhibited only minor cross-reaction. Unconjugated A-diol (10.69%) and androsterone (7.06%) also exhibited some cross-reaction. Since the extraction procedure for free steroids has been shown to remove over 99% of unconjugated steroids [9], the small cross-reaction exhibited by these unconjugated steroids did not interfere in the actual assay.

Androstanediol 17-G radioimmunoassay validation

The dose range for the RIA was 0.0667–2.134 pmol/tube. The mean half maximal binding point was 0.33 ± 0.028 pmol/tube. The slope of the dose–response curve was −2.26 ± 0.13 and the intra- and interassay coefficients of variation were 3 and 8% respectively. The value measured in blank serum (obtained from a woman with gonadal dysgenesis and Addison's disease having undetectable levels of serum testosterone, dihydrotestosterone, dehydroepiandrosterone, dehydroepiandrosterone sulfate and androstenedione) was 0.13 ± 0.07 nmol/l.

When tritiated androstanediol 17-G was added to normal male serum and the androstanediol 17-G peak from the HPLC was divided into 15-s fractions, the immunoactivity paralleled the radioactivity, indicating that the immunoreactivity in the serum sample eluted in a peak synchronous with that of [^{3}H]androstanediol 17-G (Fig. 1). Androstanediol 17-G standard, when added to blank serum, was quantitatively recovered (104 ± 3% of expected values). Values from dilutions of the androstanediol

Table 1. Cross-reactivity of androstanediol 17-G antibody

Steroid	Percent cross-reactivity
5α-Androstane-3α,17β-diol 17-glucuronide	100
5α-Androstane-3α,17β-diol	11
3α-Hydroxy-5α-androstan-17-one (androsterone)	7
5α-Androstane-3α,17β-diol 3-glucuronide	6
5α-Dihydrotestosterone glucuronide	2
Testosterone glucuronide	0
Testosterone	0
5α-Dihydrotestosterone	0
5β-Dihydrotestosterone	0
3α-Hydroxy-5α-androstane-17-one-3 glucuronide (Andros-G)	0
5α-Androstane-3β,17β-diol	0
5α-Androstane-3,17-dione	0
4-Androstene-3,17-dione	0
4-Androstene-3α,17β-diol	0
Dehydroepiandrosterone	0

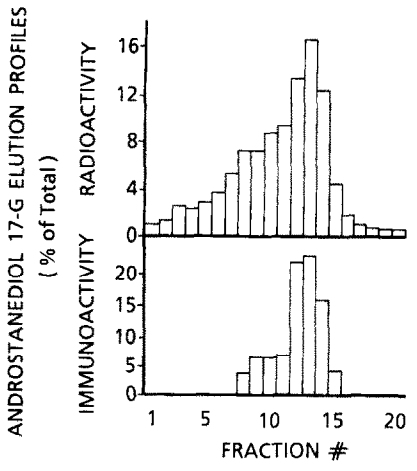

Fig. 1. HPLC elution profile of androstanediol 17-G. [9,11-^{3}H]Androstanediol 17-G was added to normal male serum, and the elution profiles following HPLC separation (0.25 min/fraction) were determined for immunoactivity (by RIA) and radioactivity. The bottom panel shows the immunologic elution profile, expressed as percent of total immunoactivity. The top panel shows the elution profile of [9,11-^{3}H]androstanediol 17-G, expressed as percent of total radioactivity. The immunoactivity paralleled the radioactivity, indicating that the immunoactivity in the serum sample eluted in a peak synchronous with that of [9,11-^{3}H]androstanediol 17-G.

17-G peak prepared from serum of normal men paralleled the standard curve.

Assay calibrations

Androstanediol 17-G standard was 106 ± 2% and the androstanediol 3-G standard was 99 ± 3% of their actual values when hydrolyzed and measured in the androstanediol assay. The measured levels in both the androstanediol 3-G and androstanediol 17-G assays were adjusted by these correction factors to allow for direct comparisons between assays.

Androstanediol-G levels in serum of normal men

Androstanediol 3-G levels in normal men were 3.3 ± 1.8 nmol/l (Table 2). Androstanediol 17-G levels were 12.9 ± 5.0 nmol/l ($P < 0.0001$). There was no significant difference between the measured total androstanediol-G levels (16.5 ± 5.2 nmol/l) and the value derived from the sum of the androstanediol 3-G and androstanediol 17-G levels (16.2 ± 5.3; $P = 0.515$). Androstanediol 17-G was the predominant isomer of androstanediol-G, being 77 ± 13% of total.

DISCUSSION

By infusing dihydrotestosterone intravenously into men, Moghissi *et al.* [14] found that androstanediol-G (39%) and dihydrotestosterone-G (30%) were the major glucuronides produced. We have shown that androstanediol 17-G is the predominant isomer formed following such as infusion [3, 4]. In this study, we have described a direct assay specific for androstanediol 17-G and have shown that this isomer is the major form of androstanediol-G present in serum. Because the distribution of androstanediol-G isomers in serum is similar to that seen following dihydrotestosterone infusion, these results are consistent with the hypothesis that androstanediol-G arises from dihydrotestosterone metabolism. However, steroids such as androsterone-G could be metabolized to androstanediol 3-G, analogous to the interconversion between androsterone and androstanediol [15, 16]. Because the levels of androsterone-G in human serum are 25–50 times greater than androstanediol 3-G levels [17], even a small percent conversion of androsterone-G to androstanediol 3-G would significantly increase androstanediol 3-G values. This would have the effect of increasing total androstanediol-G levels without a corresponding change in DHT metabolism. Under such circumstances, androstanediol 17-G would be a more reliable marker of dihydrotestosterone metabolism than would total androstanediol-G.

Using a human hepatic glucuronyl transferase assay, we have previously found that androstanediol 17-G was the major glucuronide isomer formed from androstanediol [18]. Little dihydrotestosterone-G was formed from dihydrotestosterone, suggesting that DHT is first converted to androstanediol, which is then preferentially metabolized to androstanediol 17-G. This pattern of hepatic metabolism could explain the ratio of androstanediol-G isomers found in the present study. No glucuronyl transferase activity was found in human skin (abdominal skin or foreskin fibroblasts) [18], suggesting that dihydrotestosterone formed in the skin is conjugated to glucuronic acid in other tissues, such as the liver.

Table 2. Serum levels (nmol/l) of total androstanediol-G, androstanediol 3-G and androstanediol 17-G in normal men

Subject	Androstanediol 3-G	Androstanediol 17-G	Sum	Measured total androstanediol-G	Androstanediol 17-G (% of total)
1	3.5	10.3	13.8	14.5	71
2	3.1	11.4	14.5	15.5	74
3	2.8	13.7	16.5	18.5	71
4	2.8	10.7	13.5	12.1	89
5	2.7	20.6	23.3	24.1	86
6	1.9	18.8	20.7	18.8	100
7	1.8	4.8	6.6	7.6	62
8	7.6	13.2	20.8	20.7	64
$X \pm SD$	3.3 ± 1.8	12.9 ± 5	16.2 ± 5.3	16.5 ± 5.2	77 ± 13

Paired t-test comparison between sum and measured total: $P = 0.515$.

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